

# Dose-Response Relationship in Multistage Carcinogenesis: Promoters

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Published dose-response curves of promoters of multistage carcinogenesis were selected that met the combined criteria of long study times, multiple doses, and low doses. In rat liver, 12 dose-response studies of 7 different promoters (phenobarbital, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD], clophen A-50 (a polychlorinated biphenyl),  $\alpha$ -,  $\beta$ -, and  $\gamma$ -hexachlorocyclohexane [HCH], and chloroform) were selected. These promoters were studied for 7-86 weeks and either altered hepatic foci or hepatic cancer were determined. The doses ranged from 1 ng (TCDD) to 400 mg (chloroform). In mouse skin, 10 dose-response studies of 4 promoters (12-*O*-tetradecanoylphorbol-13-acetate [TPA], anthralin, chrysarobin, and 2,6-di-*tert*-butyl-4-hydroperoxyl-2,5-cyclohexadienone [BHOOH]) were selected. In these mouse skin studies the doses ranged from 0.425 nmole (TPA) to 20,000 nmole (BHOOH) per mouse. The length of time promoters were applied to the skin varied between 15 and 60 weeks. Either skin papillomas or carcinomas were determined. The dose-response relationships are presented on the basis of moles of promoter, percentage of the fully effective promoting dose, or percentage of the acute oral rat LD<sub>50</sub>. The degree of concavity of the dose-response curves was determined. The available dose-response data are critiqued and discussed on the basis of future research needs for biologically based cancer risk assessment models.

## Introduction

As we learn more about multistage carcinogenesis, more data accumulate on the number of stages and the dose-response relationship of each individual stage. After elucidation of the initiation step of carcinogenesis, promotion of carcinogenesis was the second stage of multistep carcinogenesis identified. For decades it has been stated that initiators of carcinogenesis are probably linear in their dose-response relationship (extrapolated below the experimental range), while promoters of carcinogenesis are nonlinear and exhibit thresholds of biological response.

With the development of a mathematical two-stage model of carcinogenesis by Moolgavkar and his co-workers (*1*), the importance of the dose-response relationships of each of the individual steps of multistage carcinogenesis has become more obvious. Each important biological process in Moolgavkar's two-stage model (first mutation, second mutation, birth and death rates for initiated cells) is time and dose dependent. To use biologically based models of cancer risk assessment, we must first experimentally determine the dose-response relationship for promoters.

In this study we have compiled published dose-response relationships for promoters of carcinogenesis that meet the multiple criteria of *a*) long study times (7-86 weeks), *b*) multiple doses, and *c*) low doses selected. Without a long study time, a difference of latency might be mistaken for a difference in potency of the promoter. The resulting dose-response curves from 22 experimental studies of 11 different chemicals are examined on the basis of picomole/kilogram, nmole/mouse, percentage of the maximally promoting dose, and percentage of the acute LD<sub>50</sub>. The dose-response curves were examined for concavity (slope increases with dose in the low-dose range), experimental thresholds, slopes, and plateaus of biological effects. This dose-response study of promoters of carcinogenesis has utility for both risk assessment and the regulation of environmental chemicals.

## Rat Liver Studies

Table 1 presents the promoters, dose range, length of promotion, initiator, number and type of experimental animals, biological end point, and reference for the 12 selected experimental studies of promotion of carcinogenesis in rat liver. Although no animal species or target organs were deliberately excluded, the only studies found that simultaneously met the criteria of long study times, multiple dose, and low doses were conducted in rat liver and mouse skin. After initiation by either diethylnitrosamine (DEN), dimethylnitrosamine (DMN), or *N*-nitrosomorpholine (NNM), rats were administered various promoters anywhere between 7 and 86 weeks, depending on the individual experimental study (Table 1). In only two studies, both with

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Table 1. Experimental design of 12 dose-response studies of 7 different rat-liver tumor promoters.<sup>a</sup>

Promoter		Exposure time, weeks	Initiator	Rat strain/sex (n)	Biological end point	Reference
Chemical	Dose range, mg/kg					
Phenobarbital	4.7-75	70	DEN	S-D/M (19-23)	Carcinoma	(2)
Phenobarbital	3.84-96	86	DMN + DIET	Porton/M (8-9)	Carcinoma	(3)
Phenobarbital	0.75-75	18	NNM	Lewis/F (6)	Foci	(4)
Phenobarbital	0.78-39	26	DEN	F344/F (NA)	Foci	(5)
TCDD	0.1-100 mg	26	DEN	F344/F (NA)	Foci	(5)
Clophen A 50	0.1-10	12	DEN	S-D/F (4)	Foci	(6)
Clophen A 50	2-100	7	DEN	S-D/F (4)	Foci	(7)
Clophen A 50	0.1-50	11	DEN	S-D/F (NA)	Foci	(8)
$\alpha$ -HCH	0.1-20	20	NNM	Wistar/F (4)	Foci	(9)
$\beta$ -HCH	0.03-10	20	NNM	Wistar/F (4)	Foci	(9)
$\gamma$ -HCH	0.1-30	20	NNM	Wistar/F (4)	Foci	(9)
Chloroform	25-400	11	DEN	S-D/F (4-6)	Foci	(6,10)

Abbreviations: GGT,  $\gamma$ -glutamyltranspeptidase; HCH, hexachlorocyclohexane; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DEN, diethylnitrosamine; NNM, *N*-nitrosomorpholine; DMN, dimethylnitrosamine; S-D, Sprague-Dawley; NA, not available.

<sup>a</sup>All foci studies used GGT as a marker with the exception of one clophen A 50 study (8), which used ATPase. The Pitot et al. (5) studies of phenobarbital and TCDD employed three preneoplastic markers, GGT, ATPase, and glucose 6-phosphatase. The molecular weights of these compounds are phenobarbital (255.25), TCDD (321.96), clophen A 50 (355.7), chloroform (119.37), and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HCH (290.8).

phenobarbital (2,3), did the experiment progress to hepatic carcinoma. In the 10 remaining studies, preneoplastic foci were determined. For rat liver, the promoting chemicals were phenobarbital, chloroform,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -hexachlorocyclohexane (HCH), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and Clophen A 50 (a polychlorinated biphenyl). The doses ranged from a low of 0.0000001 mg/kg for TCDD to 400 mg/kg for chloroform, about 10 orders of magnitude.

Phenobarbital was given to male Sprague-Dawley rats in doses of 62.5, 125, 250, 500, and 1000 ppm in drinking water by Pereira et al. (2). Rats (19-23 per treatment group) were initiated with 80 mg/kg DEN by gavage. After 70 weeks of phenobarbital promotion, sections of rat liver were examined and classified into four different types of carcinomas: trabecular (the most common finding), adenocarcinoma, mixed carcinoma, and poorly differentiated carcinoma. Phenobarbital promotion at 250, 500, and 1000 ppm significantly increased the incidence of carcinoma, but the phenobarbital treatment groups at 62.5 and 125 ppm did not (Fig. 1).

The second study that proceeded all the way to full liver carcinoma was performed by Driver and McLean (3). Male SPF-Porton rats (Wistar derived) were initiated by 15 mg/kg of DMN. The animals were switched from a protein-free diet to a diet composed of 50% casein to increase mitosis just before the initiation step. The rats (10 per experimental group) were then exposed to 40, 100, or 1000 ppm phenobarbital in their drinking water for 86 weeks. Hepatocellular carcinoma was diagnosed by loss of normal liver architecture, thickened, irregular liver cell plates, and highly abnormal cellular morphology. Only the animal receiving 1000 ppm phenobarbital showed a statistically elevated incidence of liver carcinoma compared to the DEN-alone treatment group (Fig. 1). The two lower doses of 40 and 100 ppm phenobarbital were below the experimental threshold of response (Fig. 1) (3).

A third dose-response study with phenobarbital was reported by Kunz et al. (4). After initiation with 120 ppm of NNM in drinking water for 7 days, six female Lewis rats were given doses of 0.75, 7.5, or 75 mg/kg of phenobarbital for 18 weeks. The liver foci were scored for  $\gamma$ -glutamyltranspeptidase (GGT). Only the 75 mg/kg phenobarbital group showed an elevation in GGT-

positive foci (4).

Pitot et al. (5) performed a study with phenobarbital (at 0.001, 0.005, 0.01, and 0.05% in food; (Fig. 2) and TCDD (0.1, 1, 10, and 100 ng/kg/day, (Fig. 3) as promoters. Fischer 344 rats were subjected to a 70% partial hepatectomy before an oral dose of 10 mg/kg DEN was given. After 26 weeks of promotion, rat liver sections were stained for GGT, ATPase, and glucose 6-phosphatase. Altered hepatic foci were scored using all three markers. Rats promoted by the highest phenobarbital dose (0.05%) were statistically elevated in the number of altered hepatic foci, whereas rats exposed to the lowest dose of phenobarbital (0.001%) actually had a statistically decreased number of altered hepatic foci. This is experimental evidence for an antipromotional effect of a known promoter of hepatocarcinogenesis. TCDD showed the same dose-response relationship, as did phenobarbital (5). At the two lowest doses of 0.0001 and 0.001  $\mu$ g/kg of TCDD, the number of altered hepatic foci was significantly reduced. Only at the higher dose of 0.1  $\mu$ g/kg did TCDD effectively promote carcinogenesis in this two-step experimental system.

The polychlorinated biphenyl mixture Clophen A 50 has been examined by a German group in a series of three similar studies (6-8). Female Sprague-Dawley rats (4-6 female rats per treatment group) were initiated with 8 mg/kg of DEN. The promoting chemical Clophen A 50 was given either three times a week for 11 weeks (6), once a week for 7 weeks (7), or three times a week for 11 weeks (8). In these three studies the daily dose ranged from 0.1 to 100 mg/kg. In two of the studies GGT foci were scored (6,7) and in one ATPase was used as a marker (8). Five to one hundred mg/kg of Clophen A 50 significantly increased the number of altered hepatic foci, but doses below 5 mg/kg did not (Fig. 4).

The  $\alpha$ -,  $\beta$ - and  $\gamma$ -HCH isomers were used in a dose-response study of promotion in female Wistar rats (9) (Fig. 5). The doses selected were 0.1, 0.5, 2, 7, and 20 mg/kg for  $\alpha$ -HCH; 0.03, 0.2, 1, 3, and 10 mg/kg for  $\beta$ -HCH; and 0.1, 0.5, 2.5, 10, and 30 mg/kg for  $\gamma$ -HCH, the insecticidal isomer. Rats were initiated with 250 mg/kg of NNM by gavage and promotion with the three isomers of HCH started 8 weeks after initiation. After the promotional period of 20 weeks, rat liver sections were examined for GGT-positive foci. With  $\alpha$ -HCH, significant increases in GGT

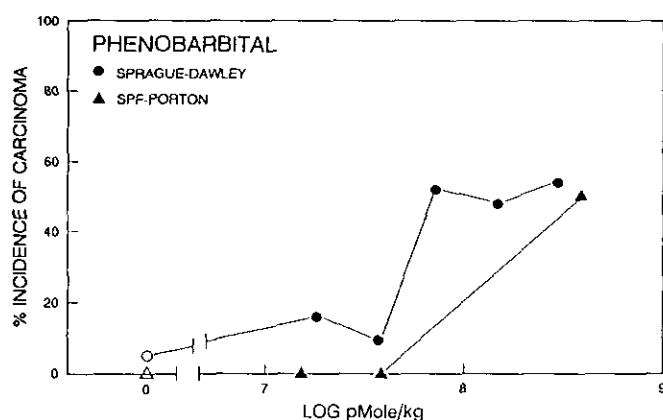


FIGURE 1. Dose-response relationship between percent incidence of hepatic cancer in male rat liver and the log of the dose of phenobarbital expressed as picomole per kilogram (2,3).

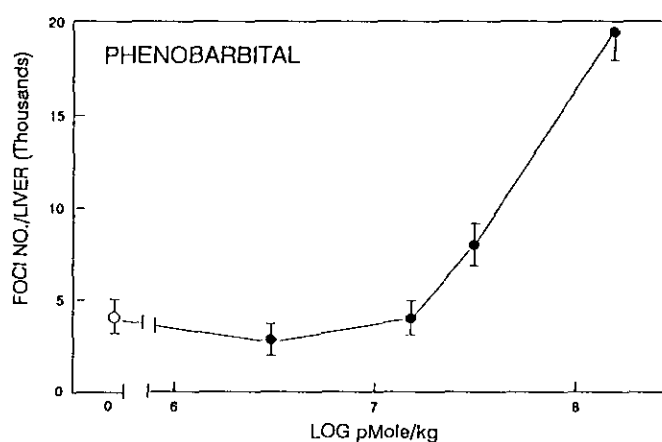


FIGURE 2. Dose-response relationship between number of female rat liver foci and the log of the dose of phenobarbital expressed as picomole per kilogram (5).

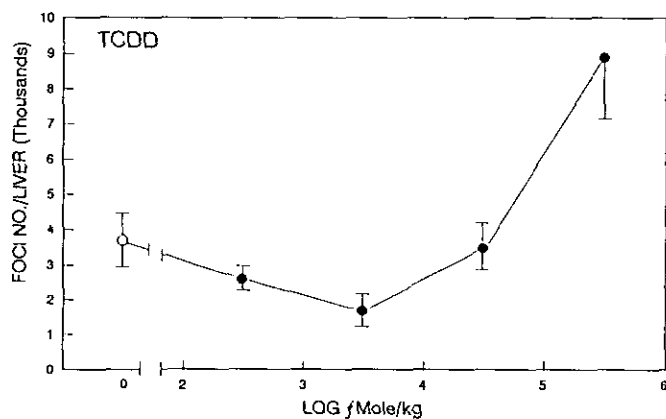


FIGURE 3. Dose-response relationship between number of female rat liver foci and the log of the dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin expressed as femtomole per kilogram.

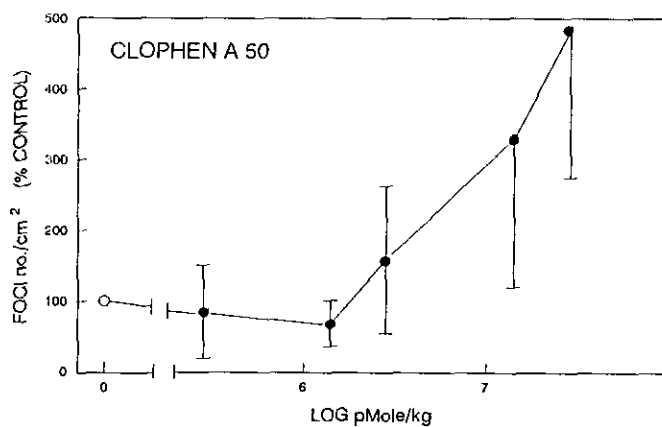


FIGURE 4. Dose-response relationship between number of female rat liver foci and the log of the dose of Clophen A 50, a polychlorinated biphenyl mixture, expressed as picomole per kilogram.

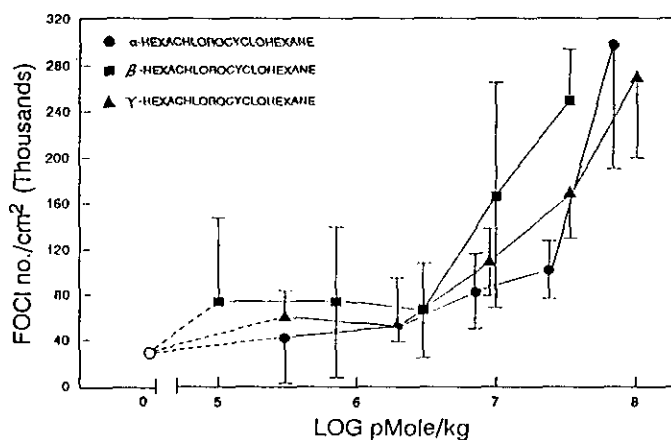


FIGURE 5. Dose-response between number of female rat liver foci (mean with SD error bars) and the log of the dose of α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH expressed as picomole per-kilogram (9).

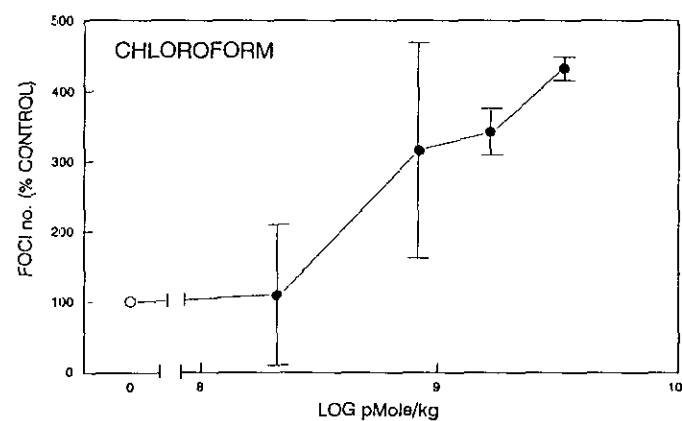


FIGURE 6. Dose-response relationship between number of weanling female rat liver foci (expressed as percentage of control values) and the log of the dose of chloroform expressed as picomole per kilogram (6).

Table 2. Experimental design of 10 dose-response studies of 4 different female mouse skin tumor promoters.<sup>a</sup>

Promoter		Exposure time, weeks	Initiator	Mice	Biological end point	Reference
Chemical	Dose range, nmole/mouse					
TPA	0-20	34	DMBA	CD-1	%P, P/M	(12)
TPA	1-20	52	DMBA	CD-1	%C	(12)
TPA	0.425-6.8	15	DMBA	SENCAR	%P, P/M	(13)
TPA	6.8	45	DMBA	SENCAR	%C	(13)
TPA	0.85-6.8	30	MNNG	SENCAR	%P, P/M	(14)
TPA	1.7-10	30	MNNG	CD-1	%P, P/M	(14)
Anthralin	50-880	27	DMBA	SENCAR	%P, P/M	(13)
Chrysarobin	50-220	30	DMBA	SENCAR	%P, P/M, %C	(13)
Chrysarobin	25-440	30	MNNG	SENCAR	%P, P/M	(14)
Chrysarobin	25-440	30	MNNG	CD-1	%P, P/M	(14)
Chrysarobin	25-440	60	DMBA	SENCAR	P/M, %C, C/M	(11)
BHTOOH	0-20,000	30	DMBA	SENCAR	%P, P/M, %C	(15)

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BHTOOH, 2,6-di-*tert*-butyl-4-hydroperoxyl-2,5-cyclohexadienone; DMBA, 7,12-dimethylbenz[*a*]anthracene; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; %P, percent of mice with papillomas; P/M, papillomas per mouse; %C, percent of mice with skin carcinomas; C/M, carcinomas per mouse.

<sup>a</sup>The molecular weights of these compounds are TPA (616.9), anthralin (226.24), chrysarobin (240.27), and BHTOOH (252.4). All studies used 30 mice per experimental group except for the BHTOOH study, which used 25 mice per group.

foci were observed at 2 mg/kg and above (Fig. 5). For  $\beta$ -HCH the highest doses of 3 and 10 mg/kg increased foci number (Fig. 5). Lower doses of  $\beta$ -HCH were not statistically significant, chiefly because of high variation among animals.  $\gamma$ -HCH showed significant promotional activity at doses of 0.1, 2.5, 10, and 30 mg/kg (Fig. 5), whereas the dose of 0.5 mg/kg  $\gamma$ -HCH did not.

The dose-response relationship for chloroform was studied by Deml and Oesterle (6,10). After initiation of Sprague-Dawley rats by 8 mg/kg DEN, various doses of chloroform (25, 100, 200, and 400 mg/kg) were administered twice a week for 11 weeks. Promotion was quantitated by GGT-positive rat liver foci. In this study, doses of 100, 200, and 400 mg/kg of chloroform increased the number of foci, but 25 mg/kg of chloroform lacked promotional activity (Fig. 6).

## Mouse Skin Studies

An outline of 10 experimental studies of four different mouse skin promoters (12-*O*-tetradecanoylphorbol-13-acetate [TPA], chrysarobin, anthralin and 2,6-di-*tert*-butyl-4-hydroperoxyl-2,5-cyclohexadienone [BHTOOH]) performed in either female CD-1 or SENCAR mice is presented in Table 2. Each individual study may express data as either percent papillomas, papillomas per mouse, percent carcinomas or carcinomas per mouse. Normally only 2 end points, most commonly percent papillomas and papillomas/mouse, are available per single experiment. In one case (11) three different biological end points are available at the same experimental time. Although one study was for only 15 weeks, the others ranged between 27 and 60 weeks of skin promotion. The doses employed ranged over about five log cycles, from 0.425 nmole/mouse for TPA to 20,000 nmole/mouse for BHTOOH. Only 2 (12,15) of the 10 experimental studies included a concurrent control group given zero dose of the promoter.

Verma and Boutwell used TPA as a mouse skin promoter following initiation of CD-1 mice (30 per treatment group) with 200 nmole/mouse of DMBA (12). This study included doses of TPA of 0, 0.01, 0.1, 1, 2, 5, 10, and 20 nmole per mouse and is the most extensive dose-response study presented in Table 2. Papillomas were determined after 34 weeks of promotion (Fig. 7); carcinomas were quantitated at 52 weeks (Fig. 8). Papillomas

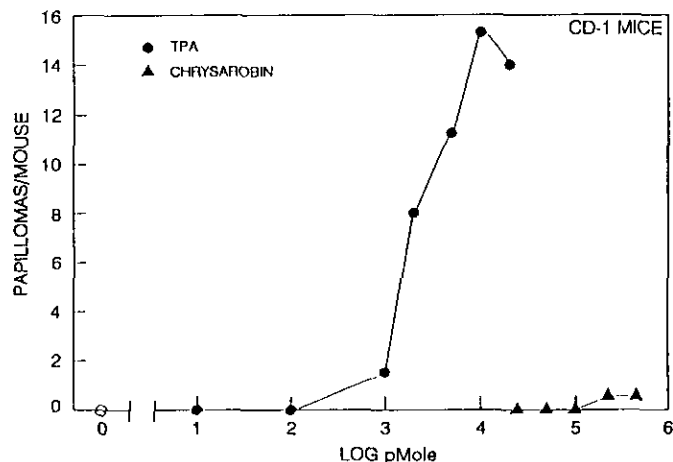


FIGURE 7. Dose-response relationship between number of papillomas/mouse and the log of the dose of either 12-*O*-tetradecanoylphorbol-13-acetate or chrysarobin expressed as picomole per CD-1 mouse (12,14).

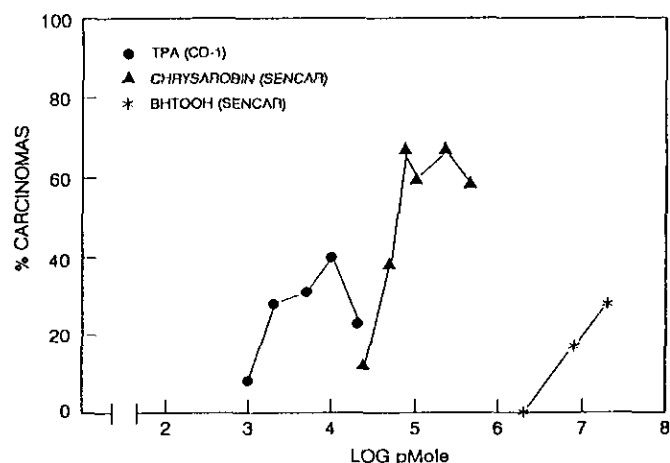


FIGURE 8. Dose-response relationship between percent skin carcinoma in CD-1 or SENCAR mice and the log of the dose of either 12-*O*-tetradecanoylphorbol-13-acetate, chrysarobin, or 2,6-di-*tert*-butyl-4-hydroperoxyl-2,5-cyclohexadienone expressed as picomole per mouse (11,12,15).

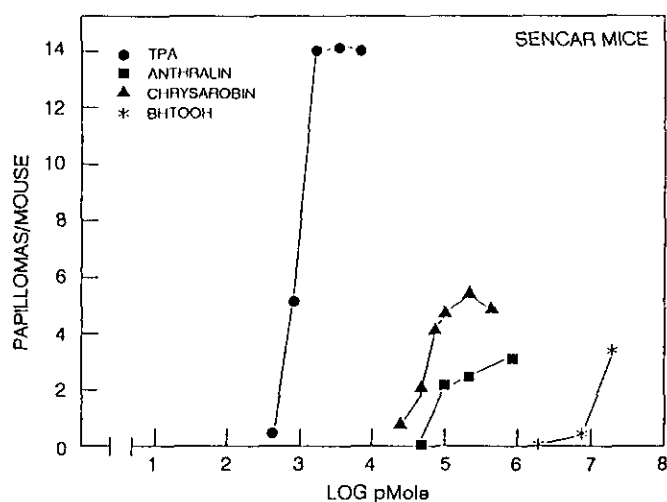


FIGURE 9. Dose-response relationship between number papillomas/mouse and the log of the dose of either TPA, anthralin, chrysarobin, or BHTOOH expressed as picomole per mouse (11,13,15).

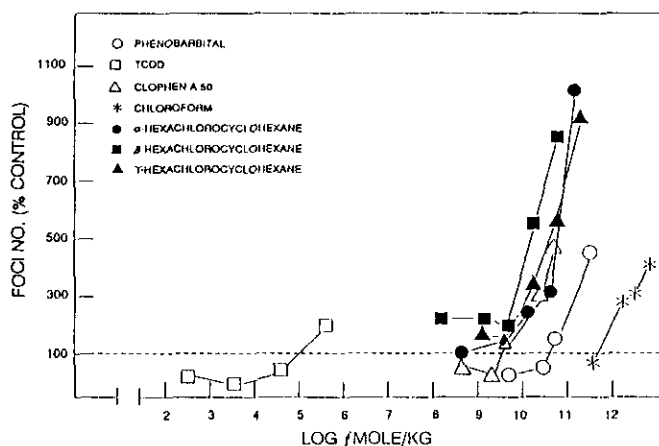


FIGURE 10. Dose-response relationship between number of rat liver foci and the log of the dose of seven different promoters of hepatocarcinogenesis expressed as femtomole per kilogram (5,6,9).

were not found after exposure to 0.01 or 0.1 nmole of TPA per mouse. All five higher doses (1–20 nmole/mouse) of TPA promoted skin papillomas (Fig. 7) and carcinomas (Fig. 8).

DiGiovanni et al. (13) studied papilloma development in SENCAR mice (30 per treatment group) initiated with 10 nmole/mouse DMBA and subsequently promoted for 15 weeks with 0.425, 0.85, 1.7, 3.4, and 6.8 nmole of TPA (Fig. 9). The percentage of mice with carcinomas was determined after 45 weeks of promotion with 6.8 nmole of TPA. Dose-response studies of papillomas were also done with 50, 100, 220, and 880 nmole of anthralin (13) (Fig. 9) and with 50, 100, or 220 nmole of chrysarobin (11) (Fig. 9). In a subsequent 30 week dose-response study, DiGiovanni et al. (14) measured papilloma formation caused by either TPA (0.85–10 nmole) or chrysarobin (25–440 nmole) in CD-1 and SENCAR mice (30 per treatment group) initiated with 2500 nmole/mouse of MNNG. The dose-

response relationship for chrysarobin-induced papillomas in CD-1 mice is shown in Figure 7.

In a related 60-week study, Kruszewski et al. (11) used 25, 100, 220, and 440 nmole of chrysarobin in SENCAR mice (30 per treatment group) previously initiated with 25 nmole/mouse of DBMA. This study determined percent papillomas, papillomas per mouse, and percent skin carcinomas at a single 60-week time point (Fig. 8) (11).

The hydroperoxide metabolite of butylated hydroxytoluene was used by Taffe and Kensler (15) in a 30-week promotional dose-response study in SENCAR mice (25 per treatment group) initiated with DMBA (20 nmole/mouse; Fig. 9). Thus, the doses chosen were 0, 2000, 8000, and 20,000 nmole of BHTOOH and are the highest molar doses of all the 10 selected studies of mouse skin promoters. The higher doses, 8000 and 20,000 nmole, promoted mouse skin carcinomas, but 2000 nmole BHTOOH did not (Fig. 8).

### Comparison of Rat Liver Preneoplastic Foci on a Femtomole per Kilogram Basis

Figure 10 shows the dose-response relationship for seven different rat liver promoters. The common Y-axis is the percentage of control foci number. The X-axis is logarithmically calibrated and ranges over 10 log cycles of promoter dose from a low of about  $10^2$  (TCDD) to a high of  $10^{12}$  (chloroform) fMole/kg body weight. Most of the seven dose-response curves are clustered fairly close together, with TCDD (16) and chloroform being the exceptions at very low and high doses, respectively. On a molar basis, TCDD is about 100,000 times more potent than the other rat liver promoters shown in Figure 10. In contrast, chloroform is about 1/100 as potent on a molar basis as the majority of rat liver promoters. This remarkable difference in molar potencies of chemicals that promote rat liver carcinogenesis may be due to three distinct mechanisms at low, medium, and high molar dose ranges. The three respective mechanisms could be high-affinity receptor mediated (for TCDD), mitogenesis or cell proliferation mediated (for the five chemicals of intermediate potency), and cell death and regenerative cell proliferation mediated (for chloroform).

### Comparisons on a Picomole per Mouse Basis

Figure 8 displays three dose-response curves for the percent carcinomas in mice exposed to different doses of either TPA, chrysarobin, or BHTOOH. Both TPA and chrysarobin show a plateau in the percent carcinomas, whereas the BHTOOH study did not have enough different doses to demonstrate this trend. The three compounds vary in molar potency by more than 1000-fold. The dose-response curves are fairly steep for all three compounds, but the TPA and chrysarobin experiments were not carried down to promoter doses that resulted in 0% carcinoma. Neither of these two studies included a control group in which mice were initiated and then given zero dose of the promoter under study. In contrast, the BHTOOH study contained both an initiated control group given zero dose of the promoter (which had 0% carcinomas) and also a 2000 pMole of BHTOOH per mouse experimental group, which also developed 0% carcinomas. In promoting mouse skin carcinomas, BHTOOH demonstrated an experimental threshold (Fig. 8).

In CD-1 mice, TPA and chrysarobin gave dose-related curves for papillomas/mouse (Fig. 7). Very few papillomas were caused by chrysarobin in the CD-1 mouse, and three experimental doses showed no effect whatsoever. Although doses of 10 and 100 pmole of TPA did not induce skin papillomas, higher doses of TPA showed a dose-response curve with a high slope. Only the TPA study included a concurrent control group exposed to the initiator and zero dose of the promoter.

In the more sensitive SENCAR mouse, dose-response curves for papillomas/mouse are available for TPA, anthralin, chrysarobin, and BHTOOH (Fig. 9). The strong promoter TPA again

showed a high slope, with a plateau at about 14 papillomas per mouse. Anthralin and chrysarobin show a lower slope, with indications of a plateau at about four to six papillomas per mouse, a level substantially below that achieved by TPA (Fig. 9). BHTOOH was tested in only three doses, and thus the slope of the dose-response curve is not really clear.

### Comparison on a Percent LD<sub>50</sub> Basis

The promotional potency normalized by the lethality potency of a chemical shows if a chemical possesses unusual promotional power without accompanying toxicities. Therefore, Figure 11 shows the number of hepatic foci graphed on the basis of percent of the chemicals' LD<sub>50</sub>/kilogram rather than femtomole/kilogram. Using this approach TCDD is not a remarkably potent hepatic promoter. Normalized on the basis of acute lethality,  $\beta$ -HCH is the most potent chemical and chloroform, again, is the least potent. Of the seven promoters, TCDD increased the hepatic foci number to the smallest extent, less than 300% of control values. Due to lack of LD<sub>50</sub> information, the four skin tumor promoters cannot be compared on a percent LD<sub>50</sub> basis.

### Comparison on a Percentage of Maximally Promoting Dose Basis

To determine if the shape of the promotional dose-response curve is fairly similar between chemicals, the maximally promoting dose of each chemical was used a normalization factor. Figure 12 displays the dose-response curves for hepatic foci number of seven promoters. For each promoter, the dose that produced the maximal promotion is set at 100% on the X-axis. Then the logarithmic X-axis is calibrated as 100, 10, 1, 0.1, 0.01, and 0.001% of the maximally promoting dose of each individual chemical. The Y-axis is foci number expressed as percentage of concurrent controls. With the exception of the three isomers of HCH, all the curves are generally the same. At 10% of the maximally promoting dose, four chemicals have fallen to nearly the no-effect level. However, at 10% of maximally promoting dose all three HCH isomers show about 300% of the control foci number. Even at a dose range of approximately 1 to 10% of the maximally promoting dose, all three isomers of HCH show a numerically higher number of foci, although these elevations were not always statistically significant. The two lowest doses that did give statistically significant increases in hepatic foci number were  $\gamma$ -HCH at 0.1 mg/kg and  $\alpha$ -HCH at 0.5 mg/kg.

### Antipromotional Dose Range of TCDD and Phenobarbital

The phenobarbital and TCDD studies of Pitot et al. (5) have shown an apparent "protective" or "antipromotional" dose range. For phenobarbital, low doses are protective in both Sprague-Dawley and Fischer 344 rats (5). At 0.001% phenobarbital in the diet, a protective effect is seen in Fischer 344 rats with respect to both number of hepatic foci and the foci volume percentages when compared with rats receiving no phenobarbital. The phenobarbital experiments were not carried out to as low levels as the TCDD experiments (Figs. 10-12). With TCDD as the hepatic promoting agent, the protective dose range

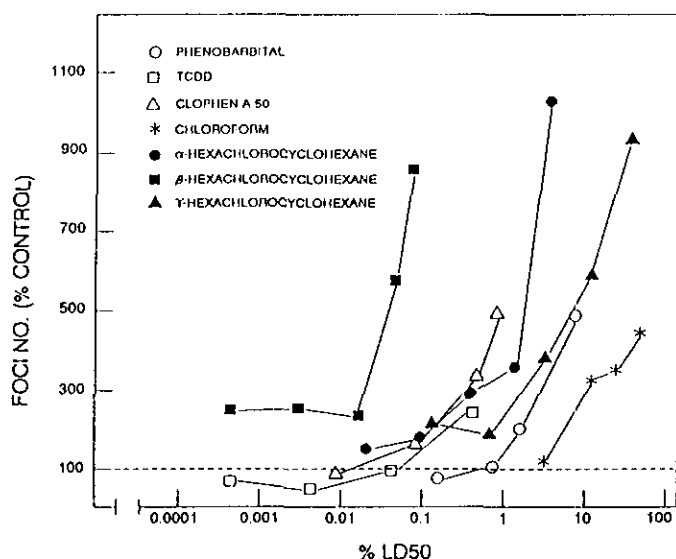


FIGURE 11. Dose-response relationship between number of rat liver foci and the percentage of the oral rat LD<sub>50</sub>(24). The X-axis is logarithmically calibrated in units of 100, 10, 1, 0.1, 0.01, and 0.001% of the LD<sub>50</sub> of each individual chemical (5,6,9).

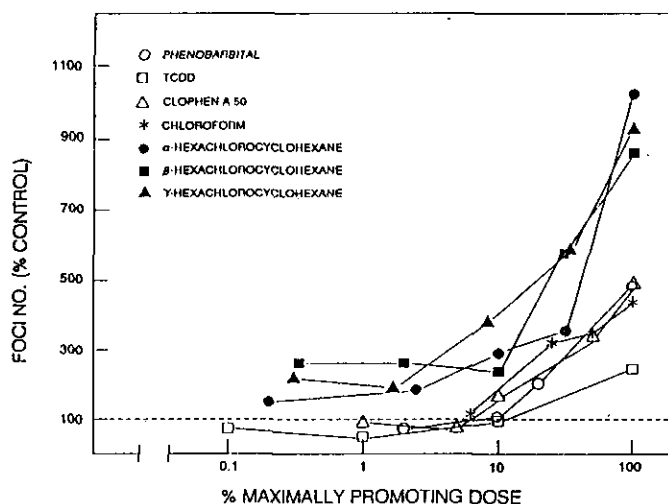


FIGURE 12. Dose-response relationship between number of rat liver foci and the percentage of maximally promoting dose of each individual promoter. The X-axis is logarithmically calibrated in units of the 100, 10, 1, 0.1, 0.01, 0.001% of the maximally promoting dose of each individual chemical (5,6,9).

included two experimental doses of 0.0001 and 0.001  $\mu\text{g/kg/day}$ . This study did not demonstrate an experimental threshold for the protective effect of phenobarbital and TCDD.

Very low dose studies of the three isomers of HCH in Wistar rats found no protective doses of HCH (Fig. 9) (9). At the lowest doses of  $\beta$ -HCH and  $\gamma$ -HCH, the experimental studies showed increases, though not always statistically significant, of about double the control numbers of hepatic foci. Thus, in the dose range below 10% of the maximally promoting dose, two chemicals (TCDD and phenobarbital) show protective effects (5), but three chemicals ( $\alpha$ -,  $\beta$ - and  $\gamma$ -HCH) do not (9).

With respect to other rat liver effects of TCDD, induction of aryl hydrocarbon hydroxylase activity does not show protective effects at low doses (17). This study used 10 different doses down to 0.0006  $\mu\text{g/kg}$ , a level corresponding to only 0.0027 % of the  $\text{LD}_{50}$  (17).

Dose-response relationships in multistage carcinogenesis have been both experimentally studied and much debated (18-21). However, in the low-dose region, the amount of experimental information available on chemically induced carcinogenesis is quite limited. Experimental approaches to the issue of dose-response relationships are limited by statistical considerations. To determine with 95% probability an animal carcinogen that causes 5, 2, 0.1, and 0.001% cancer requires 59, 149, 2995, and 29,950 experimental animals, respectively (22). This large number of experimental animals is required for equal sample sizes (treated and control) with no tumors in the control group. The largest experimental group ever run was 2109 mice in the National Center for Toxicological Research 2-acetylaminofluorene study (23).

## Experimental Thresholds

In this paper the term "experimental threshold" is defined as the dose at which no-observable 1-1 effect (NOEL) is obtained in a particular experiment. By definition, an experimental threshold should exist for every chemical in any given experimental system. The major determinants of an experimental threshold are the sample size, the standard deviation of the study parameter, and the sensitivity of the biological study parameter. However, just because an experimental threshold should exist does not mean that any particular experimental study has located the experimental threshold in the dose-response curve. The demonstration of an experimental threshold does not prove or imply the existence of a more absolute (or nonexperimental) threshold for a chemically induced health effect. The question of whether there are absolute thresholds of biological response cannot be answered with conventional dose-response experiments. Experimental thresholds are presented in this paper because *a*) they have been of some use in risk assessment procedures, *b*) they are a way to describe the dose-response curve in the important low dose region, and *c*) they show that the limit of sensitivity of detecting a biological response for a particular experimental study has been reached.

With cancer as the end point, there is good evidence with both phenobarbital in male rat liver (2,3) (Fig. 1) and BHTOOH in mouse skin (15) (Fig. 10) that experimental thresholds exist. Data of less strength that support this interpretation include TPA and chrysarobin mouse skin carcinoma data (11,12) (Fig. 8); however, no control group given zero dose of the promoter was included in these studies. Although the slope of the dose-

Table 3. Concavity and experimental thresholds of promotion dose-response relationships in rat liver.

Chemical	Biological end point	Reference	Experimental threshold?	Coefficient of quadratic dose term/SE	Statistical significance of test for concavity
Phenobarbital	Tumors	(2)	Yes	0.89	0.19
Phenobarbital	Foci/cm <sup>2</sup>	(4)	Yes	0.91	0.27
Phenobarbital	Foci/cm <sup>2</sup>	(5)	Yes	135.2	0.002*
Phenobarbital	Foci % volume	(5)	Yes	0.086	0.47
Chloroform	Foci % control	(6)	Yes	-86.0	0.996
$\beta$ -HCH	Foci/cm <sup>2</sup>	(9)	No <sup>a</sup>	-3.2	0.90
$\beta$ -HCH	Foci % volume	(9)	No <sup>a</sup>	-0.072	0.52
$\gamma$ -HCH	Foci/cm <sup>2</sup>	(9)	No <sup>a</sup>	-0.38	0.62
$\gamma$ -HCH	Foci % volume	(9)	No <sup>a</sup>	-0.007	0.50
$\alpha$ -HCH	Foci/cm <sup>2</sup>	(9)	No <sup>a</sup>	-4.11	0.92
$\alpha$ -HCH	Foci % volume	(9)	No <sup>a</sup>	0.064	0.48
TCDD	Foci/cm <sup>2</sup>	(5)	Yes	916.2	0.0003*
TCDD	Foci % volume	(5)	Yes	0.011	0.50
Chlophen A50	Foci % control	(6)	Yes	30.2 <sup>b</sup>	0.01*
Chlophen A50	Foci/cm <sup>2</sup>	(7)	Yes	2.04 <sup>c</sup>	0.15
Chlophen A50				(6.05)	(0.013)*
Chlophen A50	Foci % volume	(7)	Yes	0.015	0.50
Chlophen A50	Foci/cm <sup>2</sup>	(8)	Yes	1.63	0.18

Abbreviations: HCH, hexachlorocyclohexane; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

<sup>a</sup>By statistical tests, low doses of HCH isomers were not significantly different from controls. However, the low-dose HCH treatment groups had higher numeric values than controls, exhibited high variance, and had only four rats per treatment group. For these reasons, it was considered that experimental thresholds had not been fully demonstrated for the three HCH isomers.

<sup>b</sup>The residual SE seriously underestimated the variance in this model. The SE of the regression coefficient was estimated using the pooled mean SE.

<sup>c</sup>Not significant in the 4df model, but significant in the 5df model, with essentially the same parameter estimates.

\* $p < 0.05$ .

Table 4. Concavity and experimental thresholds in promotion dose-response relationships in mouse skin.

Chemical	Strain	Biological end point	References	Experimental thresholds	Coefficient of quadratic dose term/SE	Statistical significance of test for concavity
TPA	CD-1	% Pap	(12)	Yes	65.9	0.0048*
TPA	CD-1	Pap/M		Yes	100.6	0.0032*
TPA	CD-1	% Car		No	-2.1	0.86
TPA	CD-1	% Pap	(14)	Yes	2.14	0.14
TPA	CD-1	Pap/M		Yes	0.88	0.27
TPA	Sencar	% Pap	(13)	No	-1.2	0.78
TPA	Sencar	Pap/M		No	2.2	0.14
TPA	Sencar	% Pap	(14)	No	-5.3	0.94
TPA	Sencar	Pap/M		No	-7.6	0.96
Anthralin	Sencar	% Pap	(13)	Yes	1.1	0.23
Anthralin	Sencar	Pap/M		Yes	-0.4	0.63
Chrysarobin	CD-1	% Pap	(14)	Yes	4.2 <sup>a</sup> (2 df)	0.03*
Chrysarobin	CD-1	Pap/M		Yes	7.02 (2 df)	0.01*
Chrysarobin	Sencar	% Pap	(13)	No	0.99	0.25
Chrysarobin	Sencar	Pap/M		No	-0.47	0.64
Chrysarobin	Sencar	% Pap	(14)	No	-4.3	0.93
Chrysarobin	Sencar	Pap/M		No	-1.1	0.76
Chrysarobin	Sencar	% Pap	(11)	No	3.13	0.098
Chrysarobin	Sencar	Pap/M		No	23.4	0.014*
Chrysarobin	Sencar	% Car		No	11.0	0.029*
Chrysarobin	Sencar	Car/M		No	8.9	0.036*
BHTOOH	Sencar	% Pap	(15)	Yes <sup>b</sup>	0.26	0.42
BHTOOH	Sencar	Pap/M		Yes <sup>b</sup>	18.6	0.017*
BHTOOH	Sencar	% Car		Yes	0.18	0.44

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BHTOOH, 2,6-di-*tert*-butyl-4-hydroperoxyl-2,5-cyclohexadienone; % pap, percent of mice with papillomas; pap/M, papillomas per mouse; % car, percent of mice with carcinoma; car/M, carcinomas per mouse.

<sup>a</sup>As the response at all three of the lowest doses was zero, the dose response was evaluated for concavity over the lowest four doses.

<sup>b</sup>Although the percent papillomas and papillomas per mouse were numerically higher in the low-dose BHTOOH-treated groups than in controls, this was not a statistically significant difference.

\* $p < 0.05$ .

response curve was steep, for both TPA and chrysarobin, the lowest tested doses still had a promotional effect.

With liver foci as the end point, strong evidence for experimental thresholds exists for phenobarbital (5), TCDD (5), Clophen A 50 (6-8), and chloroform (6,10) (Fig. 10). There was no convincing experimental data for thresholds for altered liver foci demonstrated for and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HCH (9) (Fig. 10).

For skin papillomas in female SENCAR mice, strong evidence for a threshold exists for BHTOOH (15) and anthralin (13) (Fig. 9). The TPA (13) and chrysarobin (11) data are of less strength. In female CD-1 mice, strong evidence for a threshold in skin papillomas exists for both TPA (12) (two experimental doses with no papillomas) and chrysarobin (14) (three experimental doses with no papillomas) (Fig. 7). As shown in Tables 3 and 4, there were 22 cases in which it was considered that promotional experimental thresholds have been demonstrated and 19 cases in which they have not.

Thus, overall there is a sufficient amount and quality of experimental data that argue for an experimental threshold in the dose-response relationship of promoters of multistage carcinogenesis. However, only two mouse skin studies included a control group given zero dose of the promoter, and this omission limits the utility of the data in biologically based cancer risk assessment models. Unfortunately, only 5 of 22 experiments proceeded all the way to the cancer. These five studies are of phenobarbital (2,3), BHTOOH (15), TPA (12,13), and chrysarobin (13). Usually the available dose-response data on promoters of carcinogenesis are limited to altered hepatic foci and

skin papillomas. Overall the best dose-response curves available for biologically based cancer risk assessment modeling are the two rat-liver carcinoma studies with phenobarbital (Fig. 11).

## Concave Dose-Response Curves

A concave curve lies below a straight line connecting experimental points and the control values and has a slope that increases with dose for low doses. To test the concavity of the dose-response curves, data from studies of these eleven different chemicals were fit into a quadratic equation containing both a linear and a quadratic dose term. If the coefficient of the quadratic dose term is found to be positive, this is evidence for a concave dose-response curve.

As it is typical for dose-response curves to level off at higher doses, only the lowest possible sets of doses were used. The lowest  $p + 1$  doses were selected, where  $p$  is the number of parameters in the model (three for a model with an intercept and two for a model without an intercept). In two CD-1 mouse papilloma data sets (14), all doses chosen by this selection rule had zero papillomas. These two papilloma data sets were analyzed by incorporating the next higher dose.

Linear regression, weighted by the reciprocal of the squares of the estimated standard errors of the means at each dose, when they were available, was used to fit quadratic dose-responses to the end points, foci/cm<sup>2</sup>, foci as a fraction of total liver volume, foci as a percentage of control levels (minus 100%), and all the papilloma data. Models for the papilloma data and foci expressed



as a fraction of control levels were forced through the origin by not including an intercept term. A positive coefficient of the quadratic dose term was deemed statistically significant if the coefficient divided by its standard error was greater than the appropriate *t*-value for a one-tailed, one (or occasionally, two) degree-of-freedom test. In one case, the residual standard deviation was substantially smaller than the pooled standard error. In that one case the test was based on an estimate of the standard error of the quadratic coefficient that used the pooled standard error. Models for tumor prevalence were fit using quasi-likelihood methods to fit generalized linear models to the prevalence data. The standard deviation of residuals from the weighted least-square models was compared with the pooled standard error of the mean when it was available to ensure that model fit was adequate.

The results of concavity analysis of dose-response curves are expressed in Tables 3 (rat liver) and 4 (mouse skin). Linear regression analysis showed evidence for a concave curve for some of the dose-response curves of  $\alpha$ -HCH (Table 3). Statistically significant concavity was found for some of the dose-response curves of phenobarbital, TCDD, Clophen A 50, TPA, chrysarobin, and BHTOOH (Tables 3 and 4). Concave dose-response curves were not found for chloroform,  $\beta$ -HCH,  $\gamma$ -HCH, or anthralin (Tables 3 and 4). There was some degree of evidence for concavity of dose-response curves for all eight biological end points of promotion in Tables 3 and 4. As the Driver and McLean (3) phenobarbital rat tumor study could not be mathematically modeled, four experimental studies [phenobarbital (2), TPA (12), chrysarobin (11), and BHTOOH (15)] that proceeded all the way to tumors can be examined for concavity of dose-response. Of these four studies, three show some concavity in the dose-response curve, but this was statistically significant only in the chrysarobin mouse skin study (11). For six of the eight biological parameters, statistically significant concavity was also demonstrated in one or more case (only the parameters hepatic foci percent volume [ $n=6$ ] and hepatic tumors [ $n=1$ ] did not show statistically significant concavity).

## Summary

In this review of 22 published dose-response studies of tumor promoters, five scientific findings are of particular interest and utility. First, both TPA and chrysarobin show two and three experimental doses, respectively, at which zero papillomas were found. Second, the skin dose-response curves have a high positive slope (a steep curve; e. g., TPA-induced papillomas in SENCAR mice: Fig. 9). Third, phenobarbital shows the strongest evidence for a threshold of promotion of carcinogenesis in rat liver (Fig. 1). Fourth, the experimental evidence for a protective or antipromotional effect of very low doses of known hepatic promoters, TCDD and phenobarbital, is fascinating. Fifth, chloroform, a necrogenic chemical, shows an experimental threshold in the dose-response relationship with altered hepatic foci. This may be the only one of the 11 selected chemicals that acts primarily through cell death and compensatory cell proliferation.

Despite having 22 dose-response studies of 11 different promotional chemicals, there are deficiencies, gaps, problems, and limitations in the available data. Eight of these limitations are

discussed below. *a*) It is desirable that experimental studies proceed all the way to carcinoma and not stop at altered hepatic foci or mouse skin papillomas. *b*) It is desirable to use longer study times, up to the full 104 weeks normally employed in carcinogenesis bioassays. Without lifetime studies of promotion, we cannot know if a lack of response is due to insufficient dose of the promoter or to insufficient time for the promoter to act (latency period). *c*) Skin promotion experiments should include a concurrent control group given zero dose of promoter. Without this initiated control group one cannot rule out unusual and unanticipated confounding influences on an experiment. *d*) In several cases, particularly with the three HCH isomers, lower dose groups are needed to fully determine the dose-response curve. *e*) The threshold of the dose-response relationship for the protective effect of TCDD and phenobarbital on altered rat liver foci should be determined. *f*) More necrogenic chemicals should be studied in respect to their promotional dose-response relationship. Necrogenic chemicals that cause compensatory cell proliferation may be a relatively common class of chemical promoters. *g*) More data are needed in different animal species and organs. We found little dose-response data in experimental organs such as female rat liver (carcinoma studies), male rat liver (foci studies), lung, stomach, intestine, breast; and animals such as male mice, hamsters, guinea pigs, rabbits, cats, and dogs. *h*) To distinguish between various different models of promotion (e.g., linearity with log dose or linearity with arithmetic dose), it is critical that doses be selected that will provide adequate data to subsequently choose between the various possible mathematical models.

For dose-response curves of promoters of carcinogenesis to be highly useful to biologically based risk assessment models, these eight limitations should be addressed. Future dose-response research on promoters can assist in the development of biologically based risk assessment models and contribute to scientific regulation of chemicals.

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